Short Communication

Expression and Functional Analysis of CYP2D6.24, CYP2D6.26, CYP2D6.27, and CYP2D7 Isozymes[S]

ABSTRACT:

The objectives of this study were to compare the drug-metabolizing activity of human CYP2D6.24 (I297L), CYP2D6.26 (I369T), and CYP2D6.27 (E410K) allelic isoforms with wild-type CYP2D6.1 and to express the CYP2D7 protein derived from an indel polymorphism (CYP2D7 138delT) and investigate its possible codeine O-demethylase activity. Successful creation of individual cDNAs corresponding to CYP2D6*24 (2853 A>C), CYP2D6*26 (3277 T>C), and CYP2D6*27 (3853 G>A) allelic variants and CYP2D7 was achieved via molecular cloning. The corresponding proteins, CYP2D6.24, CYP2D6.26, CYP2D6.27, and CYP2D7, were expressed in insect cells by using a baculovirus-mediated expression system. All CYP2D proteins showed the empirical carbon monoxide difference spectra. We were surprised to find that the CYP2D7 protein was detected mainly in mitochondrial fractions, whereas all CYP2D6 allelic isoforms were present in the microsomal fraction. Furthermore, CYP2D7 did not produce any morphine from codeine. In contrast, CYP2D6.24, CYP2D6.26, and CYP2D6.27 allelic isoforms all showed active drug-metabolizing activities toward both codeine and dextromethorphan O-demethylation. Whereas CYP2D6.24 exhibited the highest intrinsic clearance in dextromethorphan O-demethylation (~6-fold higher than that by CYP2D6.1), it had the lowest enzyme efficiency in codeine O-demethylation (~50% lower than that by CYP2D6.1). Overall, the enzymatic consequences of CYP2D6 allelic isozymes are substrate dependent. These data would help preclinical and clinical assessments of the metabolic elimination of drugs that are mediated by human CYP2D enzyme.

Cytochrome P450 (P450) 2D6 (CYP2D6) is an important polymorphic phase I drug-metabolizing enzyme that is involved in the oxidation of 20 to 30% of clinically used drugs (Yu et al., 2004; Zanger et al., 2004; Gonzalez and Yu, 2006) and many endogenous neuroregulators or neurotoxins (Yu et al., 2003a,b, 2004). Humans deficient in CYP2D6 activity that is observed among up to 10% of whites may have an altered capacity in processing these agents (Zanger et al., 2004; Gonzalez and Yu, 2006). CYP2D6 deficiency is basically attributed to the complete defect of the CYP2D6 gene (Gonzalez et al., 1988), which has a broad range of DNA sequence variations (Zanger et al., 2004). CYP2D6 null alleles such as CYP2D6*3, CYP2D6*4, and CYP2D6*5 could result in a CYP2D6 protein that is unable to bind the substrate, a truncated apoprotein unable to bind heme, or simply no CYP2D6 protein among CYP2D6 poor metabolizers. In contrast, some CYP2D6 alleles including CYP2D6*10 and CYP2D6*17 do produce functional CYP2D6 enzymes, whereas the CYP2D6 allelic isoforms have reduced protein stability and/or drug-metabolizing capacity (Johansson et al., 1994; Yu et al., 2002; Shen et al., 2007). Whereas the enzyme functions of many CYP2D6 allelic variants, including CYP2D6*36, CYP2D6*56, CYP2D6*59, and

CYP2D6*62 (Gaedigk et al., 2006; Li et al., 2006; Toscano et al., 2006; Klein et al., 2007), have been determined, the drug-metabolizing capacity of proteins corresponding to other nonsynonymous alleles such as CYP2D6*24 (2853A>C; I297L), CYP2D6*26 (3277T>C; I369T), and CYP2D6*27 (3853G>A; E410K) (supplemental table) that were found in 0.1 to 0.3% of whites (Marez et al., 1997) remain unknown. Understanding the function of individual CYP2D6 alleles will facilitate the translation of genotype information into drug metabolism phenotype (Gaedigk et al., 2008).

Furthermore, human CYP2D locus consists of two other genes, CYP2D7P and CYP2D8P, which have been known as pseudogenes (Kimura et al., 1989; Løvlie et al., 2001). A recent report (Pai et al., 2004) described that a brain-specific variant of CYP2D7P, 138delT single nucleotide polymorphism (SNP), resulted in an open reading frame and functional CYP2D7 protein. Compared with CYP2D6, this CYP2D7 variant not only contains multiple nonsynonymous mutations that lead to over 30 different amino acid residues (supplemental table), but it also has an extra 57-base pair (bp) DNA fragment from the retention of intron 6 that encodes an additional 19 amino acids (GRRVSPGCSPIVGTHVCPV). That report (Pai et al., 2004) also stated that CYP2D7 that was expressed in Neuro2a cells produced morphine from codeine exclusively. Despite the fact that this functional CYP2D7 variant was described to occur in 50% of Indians (Pai et al., 2004), it was not observed by two independent studies (Gaedigk et al., 2005; Bhathena et al., 2007) using gene-specific assays and larger, ethnically diverse sample populations including Indians. Nevertheless, the

ABBREVIATIONS: P450, cytochrome P450; SNP, single nucleotide polymorphism; bp, base pair; CO, carbon monoxide; DXM, dextromethorphan; PMSF, phenylmethylsulfonyl fluoride; HPLC, high-performance liquid chromatography; CL$_{int}$, intrinsic clearance.

claimed function of CYP2D7 in producing morphine from codeine remains unclear.

Therefore, this study aimed to delineate the drug-metabolizing capacity of CYP2D6.24, CYP2D6.26, and CYP2D6.27 allelic isoforms and to evaluate the potential codeine *O*-demethylase activity of CYP2D7. *CYP2D6*24*, *CYP2D6*26*, *CYP2D6*27*, and *CYP2D7* cDNAs were successfully created from the wild-type *CYP2D6*1* via molecular cloning. All CYP2D isozymes were expressed in insect cells with the BAC-to-BAC approach and assayed with the carbon monoxide (CO) difference spectrometry analysis. Dextromethorphan (DXM) and codeine *O*-demethylations were determined to assess their drug-metabolizing capacities.

Materials and Methods

Chemicals. Dextromethorphan hydrobromide and dextrorphan-D-tartrate were purchased from MP Biomedicals (Solon, OH). Codeine, norcodeine, morphine, 3-hydroxymorphinan hydrobromide, 3-methoxymorphinan hydrochloride, NADPH, phenylmethylsulfonyl fluoride (PMSF), trifluoroacetic acid, and 60% of perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents used were of the highest chemical grade available.

Site-Directed Mutagenesis. The plasmid pFB2D6*1 (Yu et al., 2002) containing wild-type *CYP2D6.1* cDNA was used as a template to create other CYP2D6 allelic variants by using the QuickChange Multi-Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's recommendations. The oligonucleotides for creation of *CYP2D6*24*, *CYP2D6*26*, and *CYP2D6*27* and an extensive *CYP2D6* mutant (*CYP2D6m*) were shown in the supplemental table. After being confirmed by direct DNA sequencing analysis, correct constructs were named pFB2D6*24, pFB2D6*26, pFB2D6*27, and pFB2D6m, respectively.

Construction of Complete *CYP2D7* cDNA by Inserting the 57-Base Pair Fragment. To introduce the extra 57-bp fragment, two restriction sites (AatII and NruI) were first created on pFB2D6m plasmid by site-directed mutagenesis with a primer (5′-CACTTGGACGTCCAGCGCCGTCGCGAACAG-GAG-3′). An oligonucleotide containing the 57-bp fragment and AatII/NruI cutting sites was synthesized artificially and subcloned into the mutated pFB2D6m. The artificial AatII/NruI sites were then removed by reverse mutation with two primers (5′-CACTTGGATGTGCAGCGAGGAAGG-3′ and 5′-CCAGTCCGTGTCCAACAGGAGATC-3′). The correct plasmid containing complete *CYP2D7* cDNA was confirmed by direct DNA sequencing and named as pFB2D7.

Baculovirus-Mediated Protein Expression. Expression of CYP2D6 and CYP2D7 isozymes in *Trichoplusia ni* suspension cultures was carried out as described previously (Haining et al., 1996; Yu et al., 2001, 2002). To prepare cellular fractions, cell pellets were resuspended in homogenization buffer [320 mM sucrose, 10 mM potassium phosphate, 1 mM EDTA, and 1 mM PMSF (pH 7.4)] and lysed using French press. Microsomal and mitochondrial fractions were isolated by differential centrifugations and saved in freezing buffer [100 mM potassium phosphate, 20% (v/v) glycerol, 1 mM EDTA, and 1 mM PMSF (pH 7.4)]. P450 activity was determined according to the CO difference spectroscopy analysis (Omura and Sato, 1964).

Immunoblot Analysis. Cell lysates and microsomal and mitochondrial proteins were resolved by SDS-polyacrylamide gel electrophoresis with a 10% resolving gel, and proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were probed with a monoclonal antibody against CYP2D6 (MAB-2D6; BD Biosciences Discovery Labware, Bedford, MA), then incubated with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich), and processed with Amersham enhanced chemiluminescence detection system (GE Healthcare, Piscataway, NJ).

Enzyme Kinetics. Incubations were carried out in 100 mM potassium phosphate buffer, pH 7.4, containing 0.1 μM individual microsomal CYP2D6 or mitochondrial CYP2D7 isozymes, 0.2 μM P450 reductase, 1 mM NADPH, and 200 μl of substrate in a final volume, as described previously (Yu et al., 2002; Yu and Haining, 2006). Reactions were initiated by the addition of NADPH. DXM concentrations ranged from 0 to 30 μM, and incubations were carried out at 37°C for 5 min. Codeine concentrations ranged from 0 to 4000 μM, and incubations were carried out at 37°C for 30 min. The incubation periods were selected so that the rates of metabolite production were within the linear ranges. After being terminated by the addition of 10 μl of 60% perchloric acid, the mixtures were subject to centrifugation at 14,000*g* for 5 min. All reactions were performed in triplicate, and the supernatants were directly injected for high-performance liquid chromatography (HPLC) analyses.

Quantification of Metabolites. HPLC analyses were carried out on a Waters Alliance system (Waters, Milford, MA) consisting of the 2690 separation module, the 2487 dual λ absorbance detector, and the 474 scanning fluorescence detector. The Alliance HPLC system was controlled with Millennium 32 software. A 4.6 × 250 mm, 5 mm zorbax phenyl column (Agilent Technologies, Santa Clara, CA) was used to separate the substrates and the metabolites, similar to the method described previously (Yu et al., 2002; Yu and Haining, 2006). DXM, dextrorphan, and 3-methoxymorphinan were eluted at 22.0, 10.6, and 17.3 min, respectively. Codeine, norcodeine, and morphine were eluted at 16.9, 12.5, and 7.70 min, respectively. The calibration curves were linear from 5 to 200 pmol for each analyte. Intraday and interday coefficients of variation were less than 15%.

Data Analysis. All values were expressed as the mean ± S.E.M. Data were compared with an unpaired Student's *t* test. Difference was considered significant if the *P* value was less than 5%. Michaelis-Menten kinetic parameters, K_m and V_{max}, were estimated by nonlinear regression (GraphPad Prism version 3.02; GraphPad Software Inc., San Diego, CA). Intrinsic clearance (CL_{int}) was calculated by dividing V_{max} by K_m.

Results and Discussion

Similar to the expression of other functional CYP2D6 allelic isoforms (Yu et al., 2002), we used the BAC-to-BAC approach in expressing CYP2D6.24, CYP2D6.26, CYP2D6.27, and CYP2D7 isozymes. Immunoblot analyses with CYP2D6-selective antibody (Fig. 1, A and B) indicated the successful expression of each CYP2D protein. Because CYP2D7 consists of 19 additional amino acids, the molecular mass of CYP2D7 (~57 kD) is greater than that of CYP2D6.1 (~55 kD), which is obvious from the immunoblot (Fig. 1B). CYP2D holoproteins were monitored and assessed by measuring CO difference spectra. In contrast to the wild-type cells that did not exhibit CO difference spectrum, *CYP2D* cDNA-infected cells or cellular fractions all gave a Soret absorbance at approximately 450 nm (Fig. 1C). The other peaks at approximately 420 nm indicated the presence of CYP2D apoproteins in these samples. We were surprised to find that the P450 activity that was indicated by CO difference spectrum was mainly present in the mitochondrial fractions of cells infected with the CYP2D7 virus, whereas it was virtually absent in microsomal fractions. This is also in contrast to the presence of all CYP2D6 allelic isoforms in microsomes. Localization of CYP2D7 in mitochondria was also confirmed by immunoblot analysis (Fig. 1B). Indeed, human brain mitochondrial fractions were shown to exhibit CYP2D6-type activity (Snider et al., 2008).

DXM and codeine *O*-demethylations were used to compare the drug-metabolizing capacity of these cDNA-expressed CYP2D6 allelic isoforms. CYP2D6.1, CYP2D6.24, CYP2D6.26, and CYP2D6.27 enzymes all exhibited saturation Michaelis-Menten kinetics (supplemental figure). Apparently, CYP2D6.24 has a similar K_m value as CYP2D6.1 in catalyzing DXM *O*-demethylation, whereas it has a significantly higher V_{max} value (~6-fold) than CYP2D6.1, which leads to a higher CL_{int} value (V_{max}/K_m) for CYP2D6.24 (Table 1). However, CYP2D6.24 shows lower codeine *O*-demethylation capacity when compared with CYP2D6.1 (0.033 versus 0.061 μl/pmol P450/min), which is presumably due to its significantly higher K_m value than CYP2D6.1 (37.3 ± 7.83 versus 13.0 ± 4.36 μM). By contrast, CYP2D6.26 and CYP2D6.27 have significantly ($P < 0.05$) higher K_m values than CYP2D6.1 in catalyzing DXM *O*-demethylation, resulting in relatively lower CL_{int} values. However, there is no difference in CL_{int} in codeine *O*-demethylation by CYP2D6.26, CYP2D6.27, and CYP2D6.1 (0.087, 0.089, and 0.061 μl/pmol of
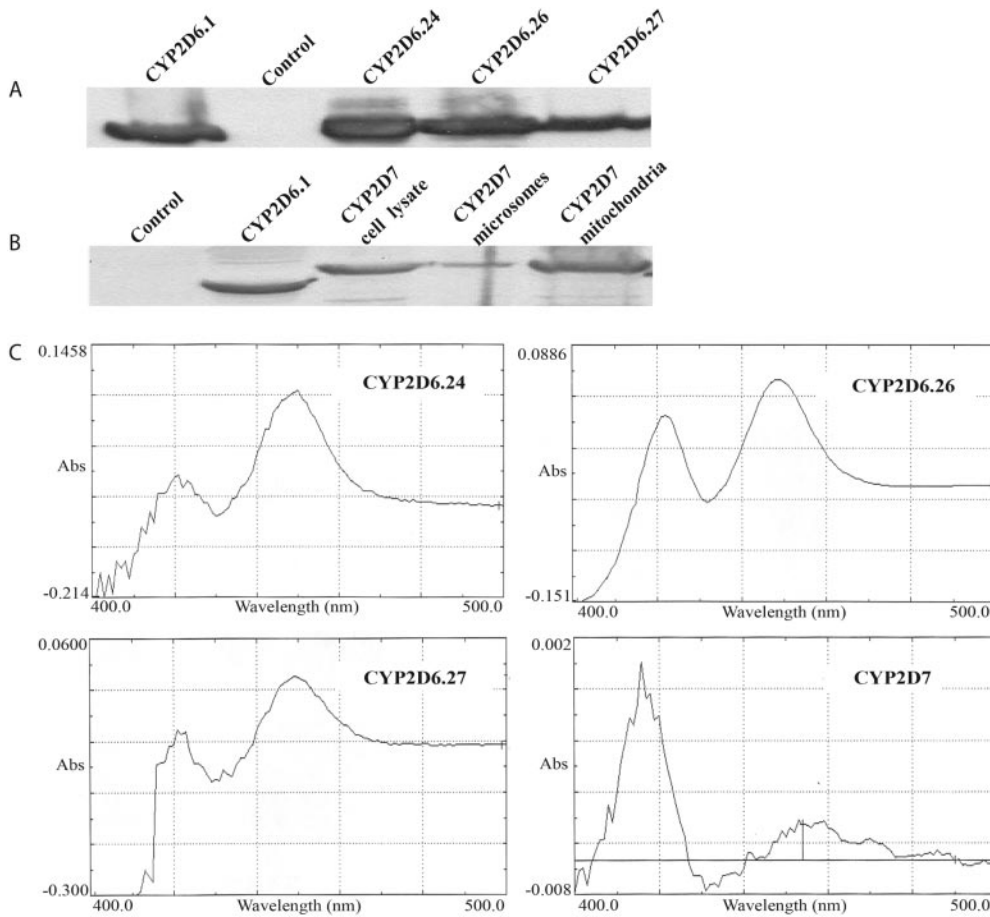
Fig. 1. Immunoblotting analyses of CYP2D6 allelic isoforms (A) and CYP2D7 isozyme (B) and CO difference spectra of CYP2D enzymes (C). *T. ni* insect cells were infected with a baculovirus carrying corresponding *CYP2D* cDNA plasmid, and cellular fractions were used for the analyses. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and probed with an anti-CYP2D6 monoclonal antibody. Controls were wild-type insect cells.

TABLE 1

Apparent Michaelis-Menten kinetic parameters for dextromethorphan and codeine O-demethylation catalyzed by CYP2D6 allelic isoforms

Values are mean $\pm$ S.E.M. of triplicate incubations at 37° C for 5 (DXM) or 30 min (codeine). DXM and codeine concentrations ranged from 0 to 30 and 0 to 4000 μM, respectively. Metabolites were quantified by HPLC with fluorescence detection. Kinetic parameters, K_m and V_{max}, were estimated by nonlinear regression.

Reaction	Allelic isoform	K_m	V_{max}	CL_{int}
		μM	*pmol/pmol P450/min*	*μl/pmol P450/min*
DXM	CYP2D6.1	0.60 $\pm$ 0.17	0.73 $\pm$ 0.04	1.22
	CYP2D6.24	0.68 $\pm$ 0.07	4.78 $\pm$ 0.09*	7.03
O-Demethylation	CYP2D6.26	3.93 $\pm$ 0.24*	2.98 $\pm$ 0.06*	0.76
	CYP2D6.27	1.45 $\pm$ 0.33*	0.63 $\pm$ 0.03	0.43
Codeine	CYP2D6.1	13.0 $\pm$ 4.4	0.79 $\pm$ 0.04	0.061
	CYP2D6.24	37.3 $\pm$ 7.8*	1.24 $\pm$ 0.06	0.033
O-Demethylation	CYP2D6.26	26.7 $\pm$ 11.4	2.37 $\pm$ 0.21*	0.087
	CYP2D6.27	10.9 $\pm$ 2.3	0.95 $\pm$ 0.03	0.089

* Significantly different from the corresponding value for CYP2D6.1.

P450/min, respectively). Overall, CYP2D6.24 exhibited the highest intrinsic clearance in DXM *O*-demethylation and the lowest intrinsic clearance in codeine *O*-demethylation. The results agree with our previous finding (Yu et al., 2002) and a recent study (Shen et al., 2007) that enzymatic efficiency of CYP2D6 allelic isoforms can vary considerably and be substrate dependent. This raises complexity in the prediction of the metabolism of a new xenobiotic agent in people carrying such CYP2D6 alleles, because their enzyme efficiencies that were determined with a known CYP2D6 substrate drug may not forecast the capacity for other agents. In addition, the inhibition potency of CYP2D6 inhibitors (Shen et al., 2007) and the metabolic capacity after induction (Felmlee et al., 2008) have been shown to be variable for different allelic isoforms and/or biotransformations. Therefore, experimental tests of allele-specific drug metabolism and

drug-drug interactions are necessary for individual biotransformations and inhibitors.

The reported functional CYP2D7 transcript (Pai et al., 2004) consists of two major SNPs, 138delT within exon 1 and g.14408G>C within intron 6, the latter of which results in the additional 57-bp fragment. However, neither mutations were observed in a variety of samples by Gaedigk et al. (2005) using a gene-specific assay. Although Bhathena et al. (2007) found the presence of 138delT SNP in 1.0% of East Asians and 0.74% of Hispanic Latinos, they did not observe the 138delT SNP in any Indians. Neither did they find the association of g.14408G>C SNP with the 138delT variants. To investigate possible activity of the claimed CYP2D7, we constructed the intact *CYP2D7* cDNA via molecular cloning and successfully expressed the functional CYP2D7 protein that was supported by CO

difference spectrum and immunoblot analysis. However, codeine *O*-demethylase activity was not observed for the mitochondrial CYP2D7 protein that was expressed in *T. ni* cells. This finding is in contrast to the high efficiency of morphine production from codeine by CYP2D7 described in that report (Pai et al., 2004), which, however, does not clarify whether CYP2D7 was present in microsomal or mitochondrial fractions of transfected Neuro2a cells. In addition, the lack of codeine *O*-demethylase activity by CYP2D7 contrasts to the active codeine *O*-demethylase activities that were consistently showed by all other membrane CYP2D6 allelic isoforms expressed with the same system (this study; Yu et al., 2002). Together, our enzymological data agree with the genotyping and sequencing findings (Gaedigk et al., 2005; Bhathena et al., 2007), suggesting that CYP2D7 is unlikely to have significant implications in drug metabolism and disposition.

In summary, CYP2D6.24, CYP2D6.26, and CYP2D6.27 allelic isoforms and CYP2D7 protein have been successfully expressed using a baculovirus-mediated system. In contrast to the deficient activity of CYP2D7, all CYP2D6 allelic isoforms are active in catalyzing codeine *O*-demethylation. Distinct enzyme efficiencies of CYP2D6 allelic isoforms toward DXM and codeine *O*-demethylations suggest that their drug-metabolizing capacities are substrate dependent. These findings shall provide helpful insight into assessing the fate of xenobiotics that are metabolized by CYP2D enzymes.

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